(6) THE SURVIVAL OF ST. LOUIS ENCEPHALITIS VIRUS IN OVERWINTERING MOSQUITOES

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INTRODUCTION (11) Jun 78

St. Louis encephalitis (SLE) virus leads all other arboviruses in causing human disease in the United States, and numerous epidemics have occurred since its initial detection in 1933. The largest being in 1975 when almost 2,000 cases were reported. The virus is maintained in nature during the summer and fall by a mosquito-bird-mosquito cycle. At least 3 mosquito species, Culex pipiens, C. nigripalpus and C. tarsalis have been incriminated as vectors. The seasonal distribution of human disease coincides with high infection rates in mosquitoes and birds during late summer and early fall, and human infections tend to be more prevalent in urban areas--especially in the eastern US where the peridomestic mosquito C. pipiens is the principal vector.

Although details of the summer and fall virus transmission cycle are relatively well known, the mechanism by which the virus persists during the interenzootic winter season has remained a mystery. In temperate regions, C. pipiens hibernate as inseminated adult females. This fact, along with the fact that this mosquito is one of the primary vectors of SLE virus during the summer and fall prompted us to test the hypothesis that SLE virus is maintained in these overwintering mosquitoes. This hypothesis has not gained wide acceptance in the past because of the belief that blood-feeding in pre-hibernating Culex mosquitoes is drastically reduced or suspended

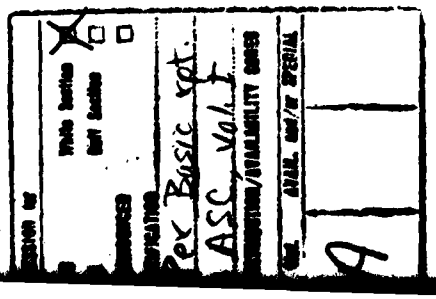
so that their chance of taking a viremic blood meal before overwintering is exceedingly small or nonexistent (1). The usual sequence of events which follow blood-feeding in C. pipiens is maturation and oviposition of eggs. Techniques have been developed (2) whereby it can be determined if a mosquito has laid eggs (parous) or not (nulliparous). Previous investigations (3) have shown that parous females are seldom found among collections of overwintering mosquitoes, and those which are found are usually observed in early winter and are presumed not to survive until spring. However, ovarian diapause (a condition of the ovaries resulting in the taking of a blood meal without development of eggs) in laboratory reared C. pipiens has been demonstrated (4). In these cases, females, experimentally induced to hibernate, took full blood meals, but in most instances, ovarian development did not follow. If this situation occurs in nature, such females, upon dissection, would show no evidence of a previous gonotrophic cycle (i.e., blood meal). Nulliparity, then, would not be proof of the absence of a pre-hibernating blood meal. This study was designed to first look for the presence of SLE virus in hibernating mosquitoes and to conduct laboratory tests to explain how these mosquitoes may have taken a pre-hibernation viremic blood meal and yet remained nulliparous.

MATERIALS AND METHODS

Virus Isolation From Hibernating Mosquitoes

The source of the mosquitoes tested for presence of virus, was abandoned ammunition bunkers of several former U.S. Army forts. Collections were made in 4 mid-Atlantic states during January and February of 1976 and 1977 (Fig. 1). The bunkers consisted of a series of rooms 3 to 5 m wide, 5 to 10 m long and 2 to 3 m high. Construction material was either steel-reinforced concrete or clay bricks. The rooms where mosquitoes were collected had condensation on both walls and ceilings.

During the 1976 studies 1,116 mosquitoes were aspirated from the damp walls and ceilings of the bunkers and transported to the laboratory where they were retained for various lengths of time. Following one day in an insectary programed for a daily photoperiod of 16 hrs daylight, 8 hrs darkness and a constant temperature and relative humidity of 26°C and 80%, respectively, 715 female mosquitoes were identified and stored at -70°C in pools of 10 mosquitoes each for virus assay. The remaining 401 mosquitoes were held for 7 days in the insectary and then pooled. No SLE isolates were obtained



from the 1976 material.

Our failure to isolate SLE virus from overwintering Culex mosquitoes collected during 1976 prompted changes in the way we handled mosquitoes for the 1977 studies (Table 1). Previous experiments with Japanese encephalitis virus (5) have shown a marked reduction in virus multiplication in mosquitoes maintained at low temperature. These data suggested that any virus present would probably be in low concentration, possibly below detectable levels, and that incubation at high temperature might be necessary for detection of virus in mosquitoes from overwintering sites. We similarly speculated that a post-hibernation blood meal might enhance the chance of recovering virus. After the holding periods in the insectary, a chicken* was placed in the mosquito cage overnight to serve as a blood meal source. The blood-engorged mosquitoes were segregated from unfed mosquitoes and maintained in separate cages until blood digestion was complete, then assayed for virus. Each mosquito pool was triturated in tissue grinders with 2 ml medium 199-Hanks salts supplemented with 20% heated fetal bovine serum, $NaHCO_3$, penicillin (500 units/ml) and streptomycin (500 µg/ml). After centrifugation for 30 min. at 475 x g in a refrigerated centrifuge, each supernatent was inoculated intracerebrally into a litter of 3-5 day old mice (0.02 ml/mouse). The remainder of each mosquito suspension was stored at -70°C to be used as necessary for virus reisolation attempts. Moribund or dead mice observed during a 14-day period were frozen at -70°C. All such suspect isolates were passaged by injecting a 20% brain suspension, in the medium described, into a second litter of mice. Results of the pre-bleedings of the chickens used to furnish blood meals for the mosquitoes eliminated any possibility that these chickens served as a virus source. SLE isolates were identified using a combination of complement-fixation (CF) and plaque reduction neutralization test (PRNT). Prototype strains used as reference SLE virus, included the Parton strain, and an isolate made from a 12 September 1975 light trap collection of Culex sp. during the SLE outbreak in Prince Georges County, MD.

*In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council.

Blood-feeding In Pre-hibernating Mosquitoes

Two characteristics of pre-hibernating adult C. pipiens females are a reduction in the blood-feeding drive and the failure of ovarian follicles to mature following a full blood meal. This latter characteristic has great importance to our understanding of the role of overwintering Culex mosquitoes in the winter maintenance of certain arboviruses. Evidence is presented which offers an explanation of how pre-hibernating female C. pipiens mosquitoes may take a viremic blood meal, yet not undergo ovarian development.

The C. pipiens mosquitoes used in these experiments were from a selfmating colony established in 1975 from hibernating females collected from Ft. Mott, Salem Co., New Jersey. Larvae for experimental material were reared in our insectary using conventional techniques, and at a temperature of 27°C and a daily photoperiod of L:D 16:8 (hr light: hr dark per 24 hr period). Since it has been established that the pupal stage is the photoperiod sensitive stage in C. pipiens, random distribution into experimental groups was made at the time of pupation. The treatment scheme used in this study is diagrammed in Fig. 2.

RESULTS AND DISCUSSION

SLE Virus Isolation

A summary of the mosquitoes collected, the number which took a blood meal and the viruses isolated during the winter of 1977 is presented in Table 1. Two SLE virus isolations were obtained from pools of 10 mosquitoes each that were collected (after the coldest periods of the winter) on 26 January and on 22 February, at Ft. Washington, MD and Ft. Mifflin, PA, respectively. Both virus isolates were obtained from mosquitoes given a post-hibernation avian blood meal. Although by no means conclusive evidence that virus isolation was contingent upon the blood meal, we did have 112 non-bloodfed mosquito pools in 1976 and 53 in 1977 in which no SLE viruses were isolated. This is compared to 2 isolates from a total of 62 bloodfed mosquito pools in 1977.

Demonstration of Increased Pre-hibernation Blood-feeding and Decreased Ovarian Diapause

The experiment reported here was designed to represent a model of fall populations of pre-hibernating female C. pipiens subjected to varying periods of warm temperatures ("Indian" summers). Our objective was to test the hypothesis that fall fluctuations in temperature would result in some females taking blood meals without the reversal of ovarian diapause. Such females would develop fat bodies, remain nulliparous, and be prepared for hibernation. The results of the blood-feeding trials and subsequent determination of ovarian development are shown in Table 2. At the time of the first blood-feeding trial only 5% of the short photophase females took a blood meal compared with 78% of the long photophase group. Of those taking full blood meals, 80% of the short photophase group had ovaries which failed to mature, compared with 0% of the long photophase group. After 3 days of warming, the percentage of females taking blood had risen to 45%, but 40% of these had undeveloped ovaries. Even after 4 days, when blood-feeding took place in 74% of the females, lack of ovarian development occurred in 25% of those taking blood. After 5 days there was no differences between the two groups. We feel that the results of these experiments provide a biologically plausible model for the natural occurrence of blood-feeding in pre-hibernating C. pipiens.

SUMMARY

Two strains of SLE virus were isolated from natural populations of hibernating C. pipiens mosquitoes collected after the coldest part of the winter in Maryland and Pennsylvania. These data suggest that these mosquitoes can be considered to be winter maintenance hosts for SLE virus. There are two possible explanations for the presence of virus in these mosquitoes. One is that they became infected through transovarial transmission, the other is that these females took a pre-hibernation viremic blood meal. We have provided an experimental basis for the latter. A significant number of females subjected in the laboratory to environmental conditions (e.g., short photophases and cool temperatures) known to produce physiological changes related to hibernation and then warmed to 25°C for various periods of time showed an increase in blood-feeding drive but still displayed ovarian diapause. Such warming periods may be analogous to situations which are common in nature ("Indian" summers). We suggest that these experimental conditions may represent the mechanism by

which SLE virus overwinters in hibernating C. pipiens. This suggests a possible strategy for the control of potentially infected vectors in the vicinity of U.S. military installations and other concentrations of human activity. Spraying of abandoned ammunition bunkers, concrete drainage tunnels, and other similar manmade structures which serve as overwintering sites for C. pipiens could attack both vector and virus at the weakest point of their life cycles.

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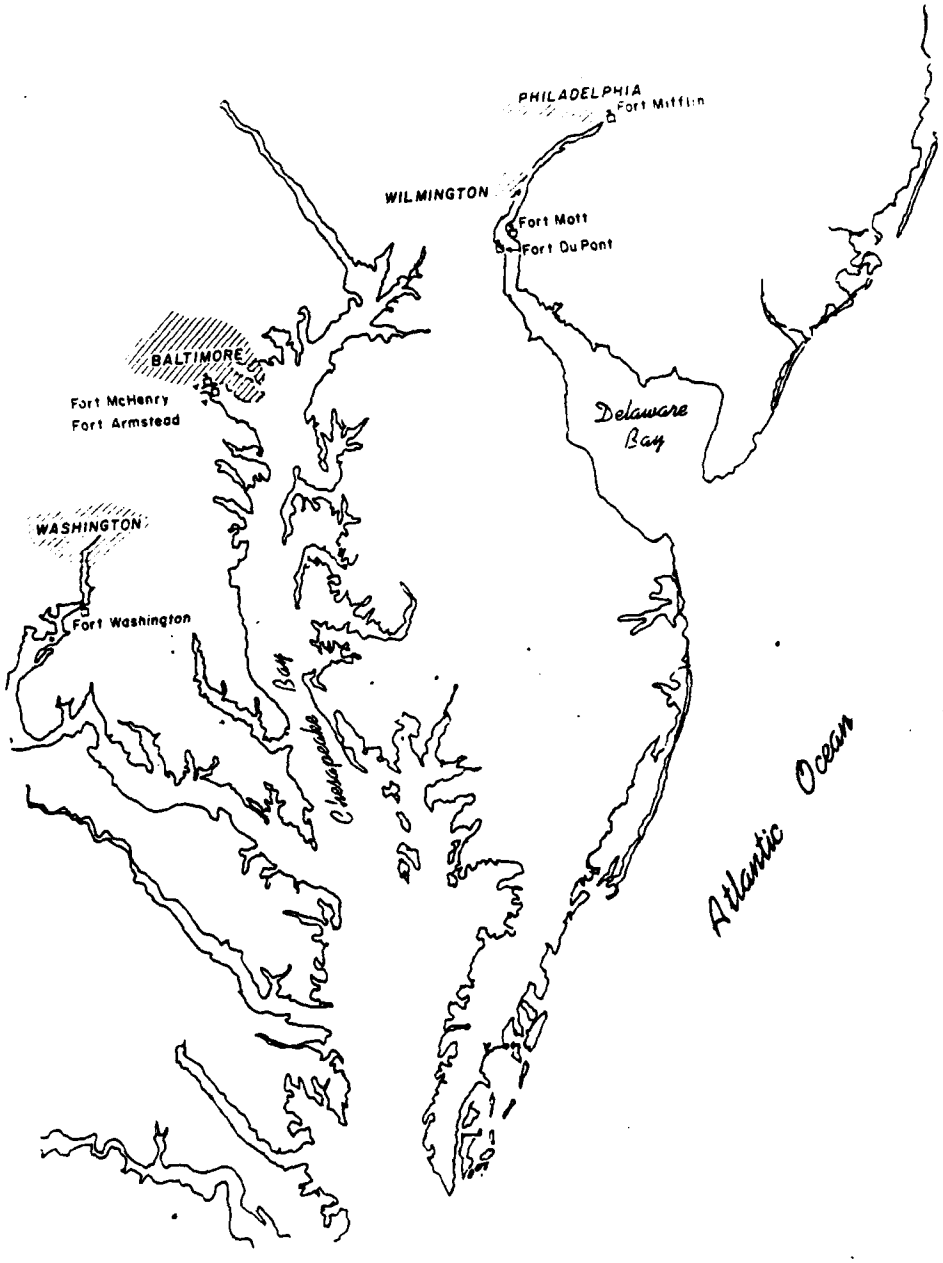

Fig. 1. Location of former U.S. Army forts where overwintering mosquitoes were collected.

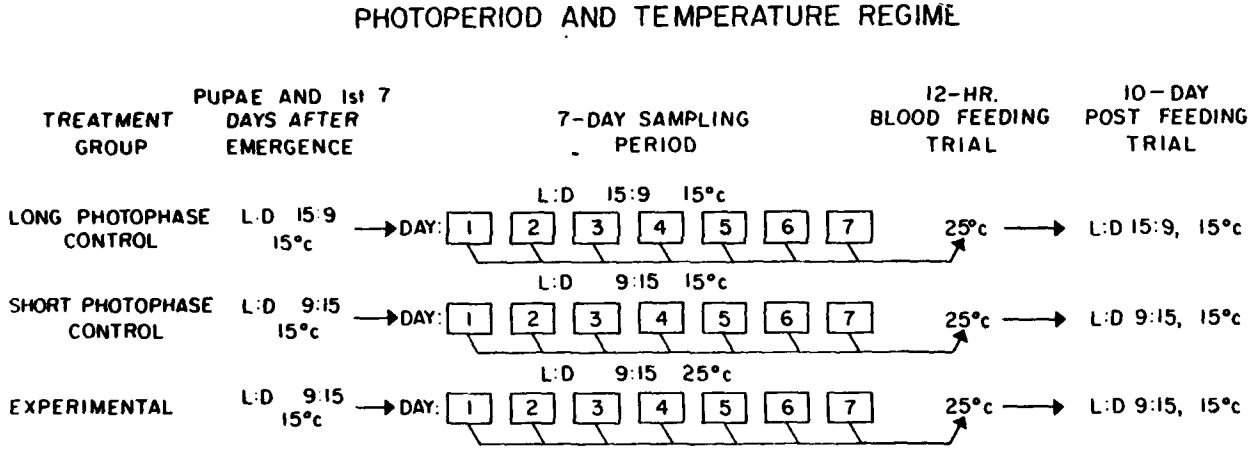

Fig. 2. Diagram of experimental treatment schedule.

Table 1. Hibernating Culex pipiens collected at abandoned Army Forts located in Pennsylvania, New Jersey and Maryland during the winter of 1977

Mosquito Collection			Laboratory Handling			
				Blood Fed		Non Blood Fed
Location	Date	Number Collected	Days held in insectary Before Blood-feeding	No. Pools Tested	No. Isolations	No. Pools Tested	No. Isolations
Ft Washington, MD	4 Jan	97	21	3	0	7	0
Ft McHenry, MD	5 Jan	9	7	0	0	1	0
Ft McHenry, MD	11 Jan	3	0	0	0	1	0
Ft Armistead, MD	12 Jan	33	20	2	0	1	0
Ft Washington, MD	26 Jan	215	20	14	1	7	0
Ft Mifflin, PA	22 Feb	406	15	25	1	15	0
Ft Mott, NJ	23 Feb	226	14	10	0	12	0
Ft Mott, NJ	1 Mar	173	20	8	0	9	0
Totals		1162		62	2	53	0

Table 2. Bloodfeeding and ovarian development in female *Culex pipiens* exposed to diapause-inducing conditions (L:D 9:15, 15°C) from pupal stage to 7th day of adult life, then warmed to 25°C for from 1 to 7 days.

	Experimental Group L:D 9:15, 15°C 25°C		Short Photophase Control L:D 9:15, 15°C		Long Photophase Control L:D 15:9, 15°C	
Day of Adult Life	Percent took full blood meal[1]	Percent[2] with undeveloped ovaries	Percent took full blood meal[1]	Percent[2] with undeveloped ovaries	Percent took full blood meal[1]	Percent[2] with undeveloped ovaries
7	5	80.0	7	71.4	78	0.0
8	18	61.1	6	83.3	70	0.0
9	21	52.4	5	60.0	79	0.0
10	45	40.0	8	75.0	73	0.0
11	74	25.7	6	50.0	82	0.0
12	61	1.6	9	55.5	77	0.0
13	42	0.0	6	66.6	76	0.0
14	71	0.0	5	80.0	-	-

[1]Each group comprised of 100 females

[2]Of those taking full blood meal